

Aqueous Extracts of Cigarette Tar Containing the Tar Free Radical Cause DNA Nicks in Mammalian Cells

Koni K. Stone, Eliezer Bermúdez, and William A. Pryor

Biodynamics Institute, Louisiana State University, Baton Rouge, Louisiana

The ability of aqueous extracts of cigarette tar to nick DNA was investigated using viable mammalian cells. Tar extracts contain a radical with a stable electron spin resonance (ESR) signal at $g = 2.0036$ characteristic of a semiquinone. The association of the tar component that carries the ESR signal with DNA was demonstrated using viable rat alveolar macrophages. The formation of single-strand DNA breaks caused by cigarette tar extracts in viable rat thymocytes follows saturation kinetics, indicating a tar component associates with DNA and then nicks it. These studies support our hypothesis that tar components that contain the cigarette tar radical can enter cells, associate with, and then nick DNA. — Environ Health Perspect 102(Suppl 10):173–178 (1994)

Key words: semiquinone radicals, autoxidation, hydrogen peroxide, catalase, superoxide dismutase, glutathione, cancer

Introduction

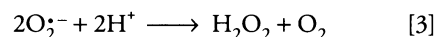
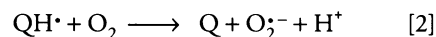
Cigarette smoking is a major cause of human lung cancer (1), and the evidence suggests that radical mechanisms are involved in causing the DNA damage that could lead to cancer (2,3). Cigarette tar contains high concentrations ($>10^{17}$ spins/gram) of stable radicals that can be observed directly by electron spin resonance (ESR) (4). At least four radicals have been identified based on their ESR spectral characteristics; the most interesting is a semiquinone in equilibrium with quinones and hydroquinones in a low molecular weight, tar matrix (2,4,5).

For each commercial cigarette smoked, up to 12 mg of particulate material (excluding nicotine and water) is deposited in the lungs of a smoker (6). This material comes in contact with pulmonary fluids that wash over it and extract the water-soluble components. Therefore, we believe aqueous cigarette tar extract (ACT) solutions are a realistic model of the mixture of chemicals that lung cells are continuously exposed to in the lungs of a smoker.

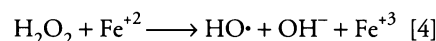
The tar fraction that contains the semiquinone radical associates with DNA (7). When calf thymus DNA is incubated

with buffer solutions containing the tar radical, the DNA recovered by precipitation contains the tar radical ESR signal, and this signal is not removed by washing the DNA (7). Aqueous cigarette tar extracts nick plasmid DNA (8), producing nicks that are not easily repairable (9); thus, error-prone mechanisms may be involved in the repair of the tar radical-induced nicks and these erroneous repairs could lead to mutations. When plasmid DNA is incubated with ACT, the resultant DNA nicking appears to follow saturation kinetics (10). Based on these preliminary DNA nicking studies, we have proposed a model in which the tar radical first associates with, and then nicks DNA (3).

It is our hypothesis (2) that superoxide and hydrogen peroxide (H_2O_2) result from the reduction of dioxygen by the hydroquinones and/or semiquinone radicals present in smoke extracts (Equations 1–3).



H_2O_2 can then be reduced to the hydroxyl (HO^\bullet) radical by metals such as iron (Equation 4), and the resulting HO^\bullet radical can then nick DNA (3,11).



The iron in reaction 4 probably is complexed to the DNA via polyhydroxyaro-

matic components in the tar (3,11). In fact, Ghio et al. have found that aqueous cigarette tar extracts complex iron *in vitro* and *in vivo* (personal communication).

We report here studies that show the tar radical component in ACT produces DNA nicks using viable mammalian cells. We also report, for the first time, ESR spectra of the tar radical in aqueous solutions and show that the number of DNA nicks produced is proportional to the amount of tar present. We also report the effects of reduced glutathione (GSH), deferoxamine, catalase, and superoxide dismutase (SOD) on the yield of DNA nicks caused by tar extracts.

Materials and Methods

Materials

Chemicals and enzymes (unless noted otherwise) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Reduced glutathione, 800 mM, pH 8.1, catalase 1 mg/ml, and SOD 1 mg/ml were all prepared just before use. Catalase was inactivated by boiling for 2 min in 1 N NaOH and then the pH was adjusted to 7.4 with HCl; SOD was inactivated by boiling for 2 min. Inactivated enzyme solutions were stored at 4°C.

Preparation of ACT

Research grade cigarettes (1R2) from the Kentucky Tobacco Research Council were smoked to a butt length of 30 mm using a standard puff profile (30 ml puff/30 sec). The tar from four cigarettes was collected

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Address correspondence to William A. Pryor, Biodynamics Institute, Louisiana State University, 711 Choppin, Baton Rouge, LA 70803-1800. Telephone (504) 388-2063. Fax (504) 388-4936.

on a Cambridge filter (a glass fiber filter that retains 99.9% of the particles larger than 0.1 micron), and the filter was extracted with 5 ml of pH 8 PBS (phosphate-buffered saline: 0.066 M phosphate, 0.85%, w/v, NaCl). After standing in the dark at 37°C for 24 hr, the solution was filtered with a 0.2- μ m filter (25 mm, polypropylene, Whatman International Ltd., Mainstone, England). This method produces ACT solutions that contain 8 to 10 mg of tar/ml of solution. An alternate method was employed to increase the tar yield. Tar deposits were washed off a Cambridge filter with approximately 15 ml of acetone until the filter was colorless, and the acetone was removed by evaporation with a stream of nitrogen. The dried residue was then resuspended in 5 ml of PBS, pH 8, in a vial and the solution was sonicated for 15 min using a Branson 2200 sonicator (Branson Ultrasonics). After standing at 37°C in the dark for 24 hr, this ACT solution was filtered with a 0.2- μ m filter (25 mm, polypropylene, Whatman). This second method yields ACT solutions that contain 20 to 27 mg of tar/ml. We believe this second method of ACT preparation yields the same kind of tar only in a higher concentration. Therefore, for each experiment the volume of ACT solution used was adjusted to keep the amount of tar constant.

Separation of ACT by Membrane Filtration

Using Centricell 30 K and 10 K membrane filters (Polyscience, Inc., Warrington, PA), three molecular weight (mw) fractions of tar were obtained: fraction 1 >30,000 mw, fraction 2 between 10,000 and 30,000 mw, and fraction 3 <10,000 mw. The amount of tar was determined to be 1.0 mg/ml, 1.6 mg/ml, and 24.4 mg/ml for fractions 1, 2, and 3, respectively. For each experiment, the volume of each fraction used was adjusted to keep the concentration of tar constant.

Isolation of Alveolar Macrophages and Thymocytes

A male Sprague-Dawley specific pathogen-free rat 92 to 94 days old (360–400 g) (Harlan Sprague-Dawley, Houston, TX) was anesthetized with phenobarbital and then sacrificed by exsanguination. The thymus was removed, placed in a normal saline solution and then mashed with tweezers to release the thymocytes. The cells were pelleted by centrifugation at 400g and resuspended in 0.87% ammonium chloride/10 mM Tris-HCl, pH 7.2,

10 mM sodium bicarbonate solution to lyse any red blood cells. After 20 min, the cells were centrifuged at 400g and resuspended in a buffered isotonic solution, pH 7.2, containing 0.25 M myoinositol, 10 mM sodium phosphate, and 1 mM magnesium chloride. The thymocytes were counted, and their viability determined using the trypan blue exclusion assay. This procedure yields 1.5 to 1.8×10^9 cells with 90 to 95% viability.

To harvest macrophages from lung, 10 ml of a normal saline solution (37°C) was instilled intratracheally into the lungs, the solution was allowed to remain for one minute, and then recovered. This was repeated three times and the lavage solutions were pooled. Alveolar macrophages were separated from the lavage solutions by centrifugation at 400g. Typically, 5 to 6 million cells with 85 to 90% viability were harvested.

DNA Nicking in Thymocytes

Thymocytes (17 million cells/tube, 1.2 ml total volume) were incubated on ice with aliquots of ACT for 90 min. For experiments involving inhibitors, the inhibitor was added to the cells prior to the incubation. The cells were then pelleted by centrifugation and resuspended in a buffered isotonic solution, pH 7.2, containing 0.25 M myoinositol, 10 mM sodium phosphate, and 1 mM magnesium chloride. The fluorescence analysis of DNA unwinding (FADU) assay has been described in detail by Birnboim (12,13). Briefly, the cells are divided into three types of tubes: T (total double-stranded DNA) tubes, in which care is taken to prevent DNA unwinding, indicate the total (maximum) amount of double-stranded DNA present. P (partially unwound DNA) tubes, which are treated with a viscous alkaline solution that allows for partial unwinding of the DNA, are used to assess DNA damage. B (blank, no double-stranded DNA) tubes, are treated with the viscous alkaline solution and then sonicated with a Branson sonifier 450, 15 pulses at 20% output (Branson Ultrasonics) to effect maximum unwinding of the DNA, and contain no double-stranded DNA. At this stage, the volume of each tube is 1.0 ml, then 1.5 ml of a 6.67 μ g/ml solution of ethidium bromide is added to each tube. The amount of double-stranded DNA in each tube is determined by measuring the fluorescence of ethidium bromide using 520 nm excitation and 590 nm emission. Three of each type of tubes, T, P, and B, are used for each determination, and the results are averaged. The amount

of double-stranded DNA remaining is calculated from Equation 5:

$$D = [(P-B)/(T-B)] \times 100\% \quad [5]$$

The amount DNA damage is determined by calculation of the damage quotient Qd, as shown in Equation 6:

$$Qd = (\log D_c - \log D_{ACT}) \times 100 \quad [6]$$

where D_c = double-stranded DNA in control cells and D_{ACT} = double-stranded DNA in ACT-treated cells (12). Using these calculations, Birnboim demonstrated a linear relationship between Qd and the amount of cobalt-60 gamma radiation that lymphocytes received (12). The amount of protection provided by the various inhibitors is calculated as shown in Equation 7:

$$\% \text{ Protection} = (1 - Qd_{\text{inhibitor}}/Qd_{ACT}) \times 100 \quad [7]$$

Binding of the Cigarette Tar Radical to DNA in Rat Alveolar Macrophages

RAM, isolated as described above, were resuspended in 2.0 ml of a solution containing 24 mM EDTA and 75 mM NaCl (the solution was adjusted to pH 7.5 with 1 N NaOH). The cells were then incubated with 1.0 ml of an ACT solution, or 1.0 ml of PBS, pH 8.0. In both incubations, the cells were allowed to sit on ice for 90 min to allow for maximum penetration of the tar into viable cells. The cell viability decreased markedly for incubations longer than 90 min. After the incubation, exclusion of trypan blue dye was used to determine cell viability to be 85 to 90%. Modification of the alkaline elution procedure (14) was used to isolate double-stranded DNA on polycarbonate (pc) filters. A Micro/Por Polycarbonate membrane (25 mm, 0.2 μ m, Spectrum Medical Industries, Inc., Los Angeles, CA) was soaked in ice-cold PBS, pH 7.4, for 20 min, and then placed in an alkaline elution funnel (Millipore, Bedford, MA). A small volume of PBS was run through the filter to check for leaks and then RAM in PBS were carefully deposited on the filter using a slow flow rate. A peristaltic pump is used to pull fluid through the filter at flow of 1 ml/min. After loading the cells on to the filter, 10 ml of a lysis solution (2% SDS, 0.025 M EDTA, pH 9.7) is added and allowed to remain in the funnel above the filter at room temperature for 60 min. This solution is then pulled through the filter and 4 ml of the lysis solution with added

proteinase K (0.5 mg/ml) is poured into the funnel and the cells are allowed to incubate at room temperature for an additional 30 min. The proteinase K solution is pulled through the filter and the filter washed with 10 ml of PBS and then air dried. These steps lyse the cells and remove protein, RNA, and any single-stranded DNA. The filter retains large pieces of double-stranded DNA. Control experiments included incubations of cells with no ACT, and incubations with ACT and no cells. The dried filters were shredded with scissors and packed into quartz tubes for examination by ESR spectroscopy.

Electron Spin Resonance Spectroscopy

ESR spectra of aqueous solutions were determined in a quartz flat cell using a Varian E-109 X-band spectrometer employing 100-kHz modulation. Radical concentrations were estimated by double integration and *g* values were determined using Fremy's salt (potassium nitroso disulfonate) as a standard for both determinations (15).

For dried samples and organic solutions, spectra were measured using a Bruker ER 100D X-band spectrometer with 100-Khz modulation frequency. The *g* values were determined by comparison with DPPH (15).

Results

Extraction of the Tar Radical into Aqueous Buffer Solutions

Previously, we have reported ESR spectra of the tar radical in organic solvents or on a filter (4). The tar radical signal is extracted into aqueous buffers at pH 8.0 and its ESR spectrum observed in aqueous buffers (Figure 1). These ACT solutions contain a broad ESR signal with a *g* value of 2.0035, which we have assigned to the tar semiquinone radical (4). This *g* value is typical of organic semiquinones (16) and organic semiquinone radicals previously have been observed in aqueous solutions at pH 8.0 (17).

DNA-Tar Radical Complexes Formed with Calf-Thymus DNA

Tar extracts contain stable semiquinone radicals that become associated with double-stranded DNA (7). In preliminary experiments, calf thymus DNA was incubated with ACT for 18 hr at 37°C and the DNA precipitated with cold ethanol. The ESR spectra of the ethanol precipitates from incubation of ACT with thymus DNA are shown in Figure 2. The treatment of ACT alone with cold ethanol does not

result in the precipitation of any material. The DNA that precipitated from ACT-DNA incubations had an ESR signal with a *g* value of 2.0044, in agreement with our previous report (7) and within the range for semiquinone radical signals (16).

Next, calf thymus DNA was incubated with ACT and the DNA was isolated on a polycarbonate filter that impedes the passage of long DNA strands (14). These incubations result in the semiquinone radical becoming retained by the filter; Figure 3 shows the ESR spectrum observed from the dried polycarbonate filter. Control incubations of ACT alone, or calf thymus DNA alone, showed no radical signal remaining on the filter. This indicates that the DNA-tar radical complex is retained on the polycarbonate filter, and there is no nonspecific retention of the tar radical on the filters in the absence of DNA.

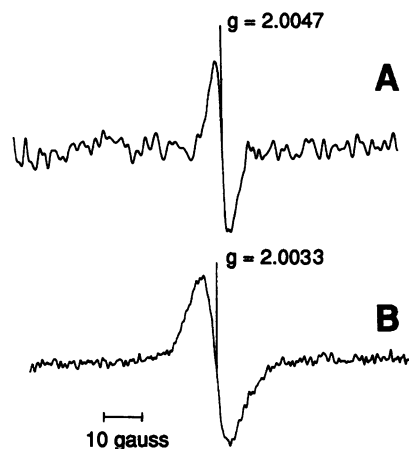


Figure 1. Comparison of the ESR spectra of the tar radical in *t*-butyl benzene (A) and the tar radical extracted into aqueous phosphate buffer at pH 8.0 (B).

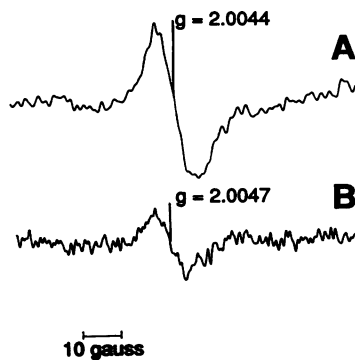


Figure 2. Comparison of ESR spectra of dried ACT and ACT co-precipitated with calf thymus DNA. (A) Ethanol precipitates of calf thymus DNA exposed to ACT, after washing off the unbound ACT. (B) Dried ACT alone.

DNA-Tar Radical Complex in Rat Alveolar Macrophages

Since the DNA-radical complex can be trapped on a polycarbonate filter, incubations of ACT with viable RAM cells were performed. RAM were incubated with ACT solutions and the DNA was immobilized on polycarbonate filters using a modification of the alkaline elution method of Kohn et al. (14). The ESR spectra of the dried filters are shown in Figure 4. The ACT solution contains a radical that associates with the double-stranded DNA in RAM, and the dried filters show this ESR spectrum. The control incubation of RAM cells alone or ACT alone show no ESR signal.

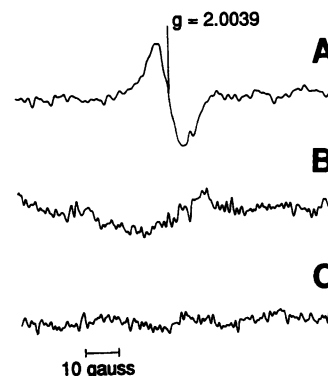


Figure 3. ESR spectra of material remaining on polycarbonate filters after the following incubations: (A) ACT + calf thymus DNA; (B) ACT alone; (C) calf thymus DNA alone.

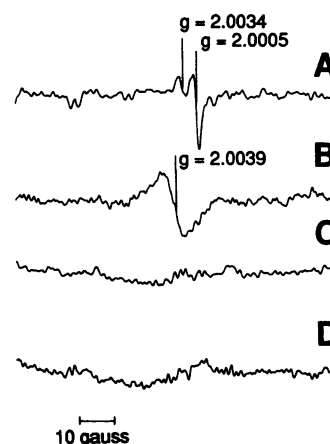


Figure 4. ESR spectra of the material bound to polycarbonate filters after the following incubations: (A) viable RAM + ACT < 10,000 mw; (B) viable RAM + ACT; (C) viable RAM alone; (D) ACT, no cells. Details of the isolation and incubations of RAM are given in Materials and Methods.

DNA Nicking by Aqueous Extracts of Cigarette Tar

The results of the FADU assay for ACT-induced DNA nicks in rat thymocytes are shown in Figure 5. The DNA damage quotient (Qd) represents the amount of DNA nicks in the rat thymocytes. (The calculation of Qd is explained in Materials and Methods.) When Qd is plotted versus the total concentration of tar in the aqueous extracts, the maximum Qd and the binding constant can be determined either from a curve-fitting program or by Lineweaver-Burk or Eadie-Hofstee plots of the data. These three analyses are summarized in Table 1, and the Lineweaver-Burk plot is shown in the insert of Figure 5. The maximum value for Qd is 98 ± 13 and the binding constant is 2144 ± 237 $\mu\text{g/ml}$ for tar extracts. These data indicate saturation kinetics, with a maximum amount of DNA damage occurring at a tar concentration of 2144 $\mu\text{g/ml}$. This is the amount of tar produced by approximately 0.23 cigarette.

Membrane filters were used to separate the ACT solution into three fractions, and these fractions were tested for DNA nicking activity. Only fraction number 3, the fraction containing material with MW less than 10,000 amu, caused DNA nicks, as shown in Figure 6. This fraction also contains the semiquinone radical that associates with the DNA in RAM, as shown in Figure 4.

Effects of Inhibitors on DNA Damage Caused by ACT Solutions

Catalase, SOD, GSH, deferoxamine, and mannitol were tested to determine if they protect DNA against nicking caused by tar extracts; the results are summarized in Table 2. Catalase and GSH protected the cells from DNA damage caused by ACT solutions. As the GSH concentration was increased, the amount of protection against DNA nicking by ACT also increased, as shown in Figure 7. Neither deferoxamine nor SOD protected against DNA nicking caused by ACT solutions. These results are summarized in Table 2.

Discussion

Studies in our laboratory with cigarette tar-induced DNA nicking were done with isolated plasmid DNA (8) or calf thymus DNA (7). We have extended our studies to determine the effects of cigarette tar in viable cells. We have also shown that the tar radical can be extracted into aqueous buffer solutions and directly observed by ESR. [We had previously reported ESR spin trap spectra of the hydroxyl radical that is produced by these ACT solutions (18).]

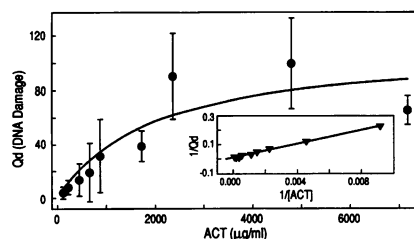


Figure 5. Plot of Qd (DNA damage quotient in rat thymocytes) versus the concentration of tar in ACT solutions. The equation for the calculation of Qd is given in Materials and Methods. The insert shows the Lineweaver Burk plot of these data.

Table 1. Comparison of data analysis for DNA nicking caused by ACT solutions.

Method of data analysis	Maximum Qd (A)	Binding constant ^a (B)
Curve-fitting (Figure 6) $f(Q_d) = A[ACT]/(B+[ACT])$	112	1968
Lineweaver-Burk plot (insert of Figure 5) $f(1/Q_d) = (B/A)(1/[ACT]) + 1/A$	102	2480
Eadie-Hofstee plot $f(Q_d) = -B(Q_d/[ACT]) + A$	80	1986
Average	98 ± 13	2144 ± 237

^aApparent binding constant units are microgram per milligram.

For DNA binding studies, we have used RAM. These cells are targets for cigarette smoke damage (19–27) and are easily isolated as a homogeneous cell population (28) from the lavage of rat lungs. We have modified the alkaline elution method of Kohn et al. (14) so that it selectively retains only double-stranded DNA from RAM on polycarbonate filters. Using this method, we are able to isolate DNA from intact cells that have been exposed to ACT and show that the tar radical binds to the DNA in these cells.

For DNA nicking studies we have employed the FADU method of Birnboim

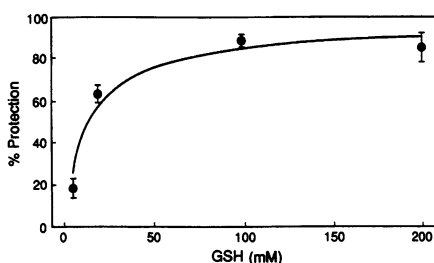


Figure 7. Plot of percent protection against ACT-induced DNA nicks versus the concentration of reduced glutathione.

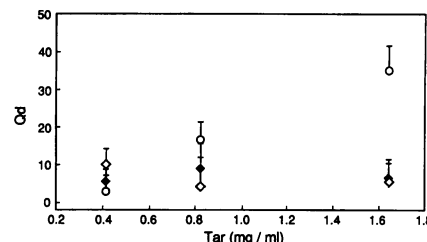


Figure 6. Plot of Qd (DNA damage quotient in rat thymocytes) versus the concentration of tar in the fractions of ACT solutions. Fraction 1 (\diamond) is $>30,000$ mw; fraction 2 (\blacklozenge) is between 10,000 and 30,000 mw; and fraction 3 (\circ) is $<10,000$ mw.

Table 2. Comparison of the effects of inhibitors for protection against DNA nicking by ACT solutions.

Inhibitor	% Protection from ACT nicking, average \pm SD	Number of experiments
Catalase	83.4 ± 12.1	6
Boiled catalase	22.3 ± 20.0	3
SOD	37.8	2
Boiled SOD	34.7	2
Catalase + SOD	97.8	2
Deferoxamine, 0.1 mM	20.7 ± 8.6	4
Deferoxamine, 0.5 mM	28.1 ± 12.8	4
Deferoxamine, 1.0 mM	26.89 ± 9.2	4
GSH, 200 mM	85.1 ± 7.1	3

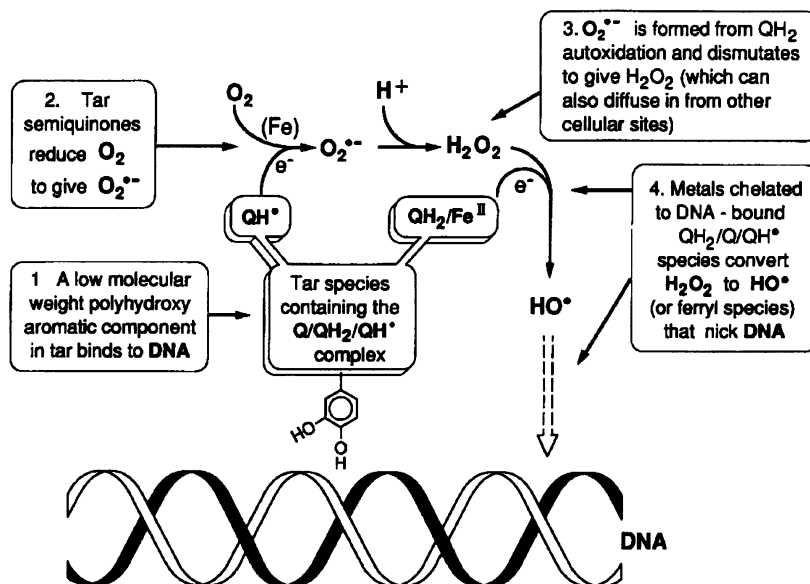
(12). This method allows the detection of DNA single-strand breaks in cells that have been exposed to ACT. For these studies we have used rat thymocytes; while these are not primary target cells for DNA damage in smokers, large numbers are easily obtained as a homogeneous cell population. We are able to routinely isolate 1.5 to 1.8 billion cells, the number required to complete a dose response curve. Therefore, thymocytes are a useful and experimentally accessible model system that we have used in our initial probing of the effect of ACT in viable cells (13).

Leanderson and Tagesson studied the ability of aqueous cigarette tar extracts to promote human polymorphonuclear leukocyte and hydrogen peroxide-induced DNA single-strand breaks in cultured human bronchiolar cells (29). The tar alone did not cause any single-strand DNA breaks. These workers used tar solutions that were 8-fold more dilute than the most dilute studied here, and apparently were too dilute to cause nicking. They also prepared their tar solutions differently than we, bubbling whole smoke through chloroform, evaporating the chloroform and redissolving the tar in 10 ml of buffer.

Nakayama et al. reported that cigarette smoke produces single-strand breaks in cul-

tured human A549 lung carcinoma cells and the number of breaks was reduced by radical scavengers (30). In their experiments, smoke extracts were prepared by bubbling the smoke from two commercial filtered cigarettes into a phosphate buffer. The authors noted the large variability (5-fold) in their results when different solutions of smoke were employed, and they attribute these difference to fluctuating conditions in trapping the smoke and the variable quality of commercial filters and cigarettes. Fielding et al. sought to determine if the tar fraction of smoke extracts caused these single-strand breaks (31). These workers used the smoke from four types of commercial cigarettes: ultra low, low, and medium tar with filters and high tar without a filter. The smoke was bubbled through buffer and the extracts then tested for DNA nicking in cultured A549 cells. These workers find no correlation between the amount of tar from a particular cigarette and the extent of DNA nicking observed, and they conclude that tar is not involved in DNA nicking. Our results indicating that aqueous tar extracts do nick DNA conflict with this conclusion. Neither Nakayama et al. nor Fielding et al. separated gas-phase cigarette smoke and tar by the standard methods we used, and both used commercial cigarettes, whereas we used standard research cigarettes.

We find that increasing amounts of tar extracts result in higher amounts of DNA damage up to a maximum (saturation) point. Using DNA isolated from rat lung and a ^{32}P -postlabeling assay for DNA adducts, Randerath et al. also found a correlation between the amount of DNA adducts and the amount of cigarette smoke extract used in the incubations (32). Their results, like ours, show that the yield of adducts reaches a saturation maximum (32). These workers found that glutathione inhibits adduct formation, and they proposed that radical mechanisms are involved in the formation of tar-DNA adducts.



Scheme 1. Model of the tar radical associating with and nicking DNA. 1) Aqueous extracts of cigarette tar contain a semiquinone/hydroquinone radical in a low molecular weight polymeric matrix that binds to DNA. 2) These semiquinones reduce oxygen to form superoxide. 3) Superoxide then dismutates to form hydrogen peroxide; or hydrogen peroxide may diffuse into the nucleus from other loci in the cell. 4) Metals chelated to the tar react with hydrogen peroxide to form hydroxyl radical (or ferryl) species that nick DNA. This figure has been modified from Pryor and Stone (11).

Cigarette smoke solutions have been shown to produce hydrogen peroxide (33). The DNA nicking caused by ACT is inhibited by catalase; hydrogen peroxide is implicated in causing DNA nicks in mammalian cells exposed to ACT. Similarly, Evans et al. showed that catalase protected against cigarette tar damage to the protein alpha-1-proteinase inhibitor (34).

Glutathione protects against DNA nicking by ACT. Glutathione is known to form covalent adducts with quinones and hydroquinones (35–37). Thus, the GSH protection of DNA may be related to the ability of GSH to add to quinones and hydroquinones and perhaps prevent the addition to and nicking of DNA, or to the well known ability of GSH to quench radical signals (38).

Aqueous extracts of cigarette tar con-

tain a tar semiquinone radical. These extracts cause DNA nicking in intact cells and this nicking follows saturation kinetics, suggesting that cigarette tar associates with DNA and then causes nicks (11). The maximum nicking occurs at concentrations of tar that are equivalent to 0.23 cigarette, using incubations of 17×10^6 thymocytes. The tar radical also associates with DNA in viable rat lung macrophages. These results show that tar components are capable of entering a viable cell, penetrating the nucleus, and interacting with DNA, and support our model of the tar radical associating with, and then nicking, DNA (11) as shown in Scheme 1. This provides further evidence that the cigarette tar component containing the free radical is involved in the DNA damage and subsequent carcinogenicity of cigarette tar.

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